DIVALENT CATIONS AND COMPLEX FORMATION BETWEEN TISSUE THROMBOPLASTIN AND FACTOR VII

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Summary. The complex formation between tissue thromboplastins from different tissues and human factor VII was investigated in the presence of calcium and other divalent cations. The calcium concentration needed to obtain full complex formation was dependent on the origin of the thromboplastin preparation. Results indicate that a specific calcium binding site on thromboplastin is involved in the complex formation and subsequent activation of factor VII. The binding of calcium by this site is stronger than the binding by citrate, indicating that citrate does not prevent activation of factor VII by tissue thromboplastin.

Strontium, manganese, cobalt, magnesium, barium and zinc were decreasingly effective in the order listed.

Calcium ions are involved in several stages of the blood coagulation mechanism, among which is the activation of factor VII by tissue thromboplastin. Calcium is necessary for the formation of a sedimentable thromboplastin--factor VII-calcium complex in which factor VII is transformed into an active form (1-6). In studies on the activation of factor VII, efforts to recover the active factor by dissociation of the bovine thromboplastin-bovine factor VII complex with various reagents were without success (3,4). An inactive factor VII which could be completely reactivated by tissue thromboplastin was obtained after complete degradation of the phospholipids in the human thromboplastin-human factor VII-calcium complex by phospholipase C (5).

We studied the binding of calcium by tissue thromboplastin and the complex formation between thromboplastin, factor VII and calcium in order to obtain insight into the nature of this complex formation. In addition, the

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capacity of other divalent cations to function in this complex formation was investigated.

Materials and Methods

Human brain thromboplastin was prepared and purified according to Hecht and Oosterbaan-Van Lit (7). Porcine lung thromboplastin was obtained as described by Chargaff et al. (8), with some modifications (9). Factor VII was purified from fresh human plasma (10). The divalent metal ions were used as their chloride salts. All preparations were made in 57 mM HEPES[1] adjusted with TRIS to pH 7.35 (HEPES-TRIS buffer).

Calcium binding was measured at 25°C by: a) the spectrophotometric method with murexide (0.14 mM) according to Ohnishi and Ebashi (11) using a Perkin-Elmer Model 356 double-beam, double-wavelength spectrophotometer; and b) the [^{45}Ca]calcium isotope method in which thromboplastin samples were suspended in solutions containing a constant amount of [^{45}Ca]calcium and increasing concentrations of unlabeled calcium. After sedimentation of thromboplastin by centrifugation, the radioactivity in the supernatant was measured with a Packard Tricarb liquid scintillation counter and used for calculation of free and bound calcium. The results were plotted according to Scatchard (12).

Factor VII activity was estimated according to the method of Lechner and Deutsch (13) as modified by Hemker et al. (14). Tissue thromboplastin activity was determined in a two-stage assay (9).

Mixtures of tissue thromboplastin and factor VII were prepared by taking about 20% excess of factor VII on an activity basis as determined in the two-stage thromboplastin assay by using limiting thromboplastin/excess factor VII and excess thromboplastin/limiting factor VII mixtures.

DFP treatment was accomplished by incubating the preparation under investigation in the presence of 2.5 mM DFP for 90 min at pH 7.35 and room temperature. The residual activity was determined after dialysis for 16 hours against the HEPES-TRIS buffer at 4°C.

Results and Discussion

Tissue thromboplastin and human factor VII, tested separately or together, were completely insensitive to DFP as were thromboplastin plus calcium or factor VII plus calcium. However, DFP inactivated factor VII in the thromboplastin-human factor VII-calcium complex. These results confirm preliminary observations of Nemerson (3). This different behaviour of non-activated and activated factor VII towards DFP was applied as a tool to study the conditions for the calcium-dependent complex formation.

The residual factor VII activity in mixtures of human factor VII and different, purified thromboplastins at increasing calcium concentration after DFP treatment is shown in Fig. 1. The results demonstrate that a much lower

[1]Abbreviations: HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TRIS = Tris-(hydroxymethyl)-aminomethane; DFP = Diisopropylfluorophosphate.

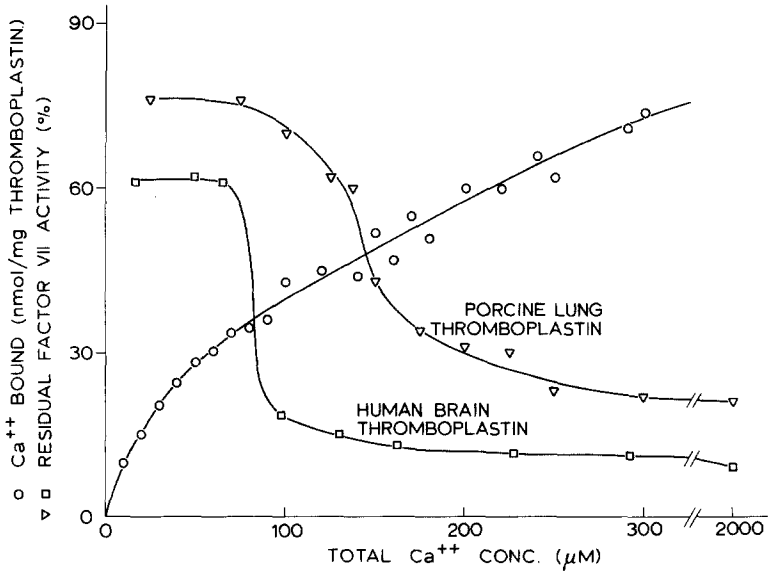

Fig. 1. Residual factor VII activity of mixtures of human factor VII and different thromboplastins in the presence of increasing calcium concentration after DFP treatment and the amount of calcium bound by porcine lung thromboplastin at these calcium concentrations.

calcium concentration than normally present in plasma is sufficient to produce complex formation. The calcium concentration ranges in which full complex formation occurs are rather narrow, especially for human brain thromboplastin. It is striking that this concentration range varies with the thromboplastin used. This suggests that complex formation is mainly governed by the binding of calcium to the particular thromboplastin.

Consequently, the binding of calcium by the porcine lung thromboplastin was studied in detail by two different techniques. The plots of the spectrophotometric method reveal the presence of a strong and a weak calcium binding site and probably an intermediate form (Fig. 2). The dissociation constants of the strong and weak calcium binding sites are 5×10^{-6}M and 2×10^{-4}M, respectively, as calculated directly from the Scatchard plot. However, binding sites which bind calcium less strongly than does murexide (dissociation constant 2.2×10^{-3}M) are not detected by this method. Estimation of calcium

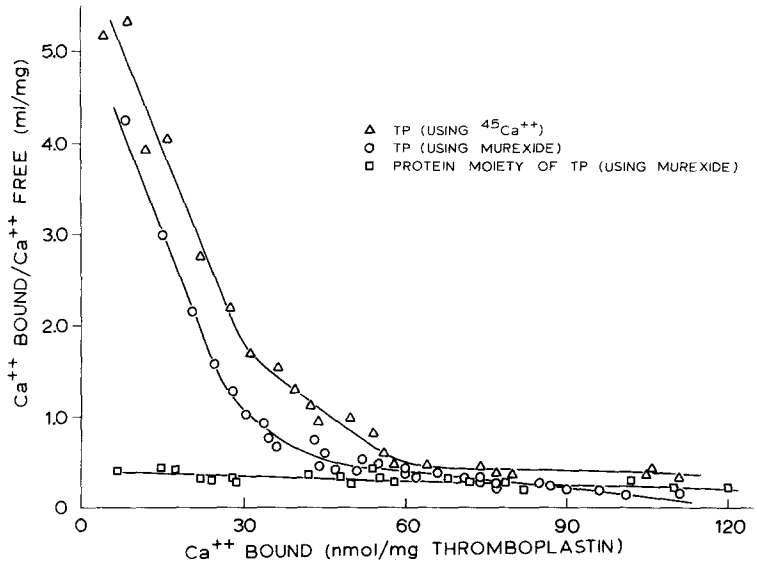

Fig. 2. Scatchard plots of calcium binding at pH 7.35 by porcine lung thromboplastin (1 mg/ml) and its purified protein moiety (1 mg/ml).

binding by using [^{45}Ca]calcium showed very clearly the strong and the intermediate binding site (dissociation constants 5 x 10^{-6}M and 3 x 10^{-5}M), while the horizontal part of the Scatchard plot indicates the presence of at least two types of weak binding sites, possibly together with nonspecific adsorption (Fig. 2). Thus, porcine lung thromboplastin contains three calcium binding sites which bind stronger than does murexide and one or more binding sites which are weaker than that of murexide. The binding of calcium by the protein moiety of thromboplastin revealed that this part of the lipoprotein binds calcium very weakly or only nonspecifically (Fig. 2).

Fig. 1 shows that complex formation is most evident when 40 to 50 nMol of calcium are bound per mg of porcine lung thromboplastin. Fig. 2 shows that the calcium binding site with the dissociation constant of 3 x 10^{-5}M is occupied at these values and could be involved in the complex formation with factor VII. This binding site has a higher affinity for calcium than that of murexide and citrate (dissociation constants 2.2 x 10^{-3}M and 6.75 x 10^{-4}M, respectively). Thus, complex formation between thromboplastin, factor VII and

Table I. Effect of DFP on mixtures of porcine lung thromboplastin, human factor VII and calcium in the presence of murexide and citrate.

AGENT ADDED	RESIDUAL FACTOR VII ACTIVITY* (%)	
	Untreated	After DFP treatment
None	80	25
Murexide**	71	24
Citrate**	68	27

*Estimated after dialysis against the HEPES-TRIS buffer.
**Concentration: 0.3 mM.

Table II. Effect of DFP on a mixture of porcine lung thromboplastin and human factor VII in the presence of different divalent cations.

CATION ADDED**	RESIDUAL FACTOR VII ACTIVITY* (%)	
	Untreated	After DFP treatment
Calcium	112	20
Strontium	108	26
Manganese	102	35
Cobalt	80	50
Magnesium	108	51
Barium	42	54
Zinc	87	81

*Estimated after dialysis against the HEPES-TRIS buffer.
**Salt concentration: 0.3 mM.

calcium, and subsequent activation of factor VII would not be expected to be prevented by murexide and citrate. This was confirmed experimentally as shown in Table I. Therefore, the residual activity curves in Fig. 1 can be considered as the titration curves for the calcium binding sites which are involved in the complex formation. The difference between the curves of the two thromboplastins suggests that the affinity of this calcium binding site of human brain thromboplastin is markedly higher than that of porcine lung thromboplastin. These findings may offer an explanation for most of the different, unsuccessful attempts to dissociate the bovine and human thromboplastin-fact-

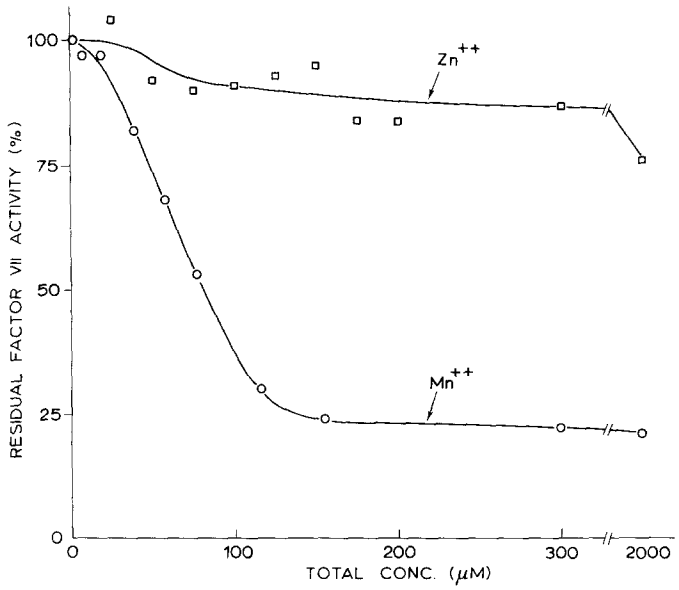

Fig. 3. Residual factor VII activity of mixtures of human factor VII and porcine lung thromboplastin in the presence of increasing manganese or zinc concentration after DFP treatment.

or VII-calcium complexes by the calcium chelating agents used (7,8,9) as well as for the "reacting system that uses a very small amount of calcium, and that activates factor VII" observed by Honorato et al. (15). Furthermore, they indicate that the use of citrate as an anticoagulant is not suitable for the prevention of the activation of factor VII by thromboplastin released by tissue damage during the collection of blood.

The results of the experiments in which the capacity of other divalent metal ions to participate in the complex formation was compared with that of calcium are given in Table II. After DFP treatment, the lowest residual factor VII activity, so the best formation of an active complex, was obtained with calcium, while strontium and manganese were also functional. Zinc was the least capable ion. Some ions were studied over a broad concentration range to determine whether the values obtained at one concentration are representative. Fig. 3 shows that a zinc concentration of even 2 mM barely promotes active complex formation. The curve for the reasonably functional manganese

resembles much the one for calcium shown in Fig. 1.

These results demonstrate that specific physicochemical properties are required for the formation of an active complex between tissue thromboplastin and factor VII with respect to the necessary divalent cation and that calcium is by far the best cation.

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